

A Simple Method to Monitor Growth of Bacterial Populations in Leaf Tissue

Greg Bertoni and Dallice Mills

Department of Botany and Plant Pathology, Oregon State University, Corvallis 97331.

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ABSTRACT

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This paper describes a simple, reproducible, and inexpensive technique to accurately monitor the growth rates of phytopathogenic bacteria in plant leaves. After growing bacterial cultures to 10^8 colony-forming units (cfu) per milliliter and diluting 100-fold in phosphate buffer, a disposable syringe was used to consistently introduce approximately 10^3 cfu into discrete sites on the leaf blade for subsequent random sampling on days 0, 1, 3, and 6 postinoculation. Leaf disk samples containing the inoculated area were then taken with a 6-mm cork borer, macerated in a unique tissue grinding apparatus devised for rapid and efficient isolation of bacteria from these samples and bacterial populations per sample determined using a micro-

plating method that required a minimum of time and materials. This technique was used successfully in bean leaves to compare the in planta growth dynamics of two wild-type strains of *Pseudomonas syringae* pv. *syringae*, the causative agent of brown spot disease of *Phaseolus vulgaris*, and two mutant strains of lessened virulence derived after Tn5 mutagenesis of one of the wild-type strains. A clear difference in growth rates and plateau populations, correlating with symptom severity, was observed between the two wild-type strains, and altered growth patterns compared with the parental were demonstrated for the two mutant strains.

In phytopathological studies, it is important to understand the growth dynamics of the pathogen in resistant or susceptible host tissue. When studying genetic and molecular aspects of pathogenicity, it is also crucial to quantify the growth potential of mutated strains derived from a wild-type pathogenic organism. Important growth parameters to be defined for these strains are: the qualitative ability to multiply in host tissue, the quantitative growth rates and maximum population levels, and the ability of the bacteria to spread and colonize fresh tissue.

Other researchers have studied the growth of bacterial foliar pathogens and related mutants using a variety of inoculation and sampling methods (3,4,6-8,10-15). Problems with these methods

may include application of imprecise numbers of bacteria, nonuniform sampling units, lengthy processing times in a variety of expensive tissue grinders, or the possibility of pathogen release into the environment either during inoculation or after sampling.

This paper describes an inoculation and sampling method that can circumvent these problems and will demonstrate its usefulness in defining growth patterns of four strains of *Pseudomonas syringae* pv. *syringae*. Two of these strains (J900, PS9020) are wild-type pathogens of *Phaseolus vulgaris* L., and two (PS9021, PS9024) are Tn5-induced mutant strains of PS9020 showing reduced virulence in bean leaves (1,9). Determination of the growth patterns of these mutants, in conjunction with further genetic information concerning the sequences disrupted by Tn5, should enable a clearer understanding of exactly what genotypic factors are required by a pathogen for the ability to multiply in host tissue and cause disease symptoms.

MATERIALS AND METHODS

Bacterial strains. A list of *P. s. pv. syringae* strains used for this study is provided in Table 1. These strains were stored and cultured in MaNY medium (2) supplemented with appropriate antibiotics purchased from the Sigma Chemical Company, St. Louis, MO (streptomycin sulfate—50 µg/ml, kanamycin sulfate—50 µg/ml). Master cultures stored in broth containing 15% glycerol were kept at -70 C and were used to prepare slant cultures for storage at 4 C. Bacteria from these slant cultures were streaked onto agar plates to obtain single colonies that were subsequently used for inoculation experiments.

Plants. Seeds of the susceptible bean cultivar *P. vulgaris* 'Eagle' provided by Asgrow Seed Company, Twin Falls, ID, were sown in a 1:1:1 mixture of peat moss, perlite, and vermiculite in a growth chamber at 26 C. Plants were watered daily and after emergence were fertilized weekly with Hoagland's solution (5). Recently expanded primary leaves were used for inoculation when the plants were 10–13 days old.

Inoculum preparation. For each strain, 5 ml of MaNY broth plus appropriate antibiotics in a 13- × 100-mm screw-cap Kimax culture tube was inoculated from a single colony and grown overnight in a shaking incubator at 28 C, 275 rpm. Growth of bacteria in this type of tube could be closely monitored by direct spectrophotometric measurement, eliminating the risk of contamination. The following day, 0.1 ml of this overnight starter culture ($A_{600nm} = 1.2-1.5$) was used to inoculate a second, identical tube. Each culture was grown to a density of 10^8 colony-forming units (cfu)/ml, represented by an A_{600nm} of 0.20–0.30, depending on the strain. After 1 ml of cells was pelleted by top-speed centrifugation for 2 min in a Beckman Microfuge II (Beckman Instruments, Inc., Palo Alto, CA), the medium was removed by aspiration, and the pellet was resuspended to its original density in 1 ml of K buffer (0.01 M K_2HPO_4/KH_2PO_4 , pH 7.0). The actual inoculum was prepared by diluting this suspension 100-fold in K buffer to a density of 10^6 cfu/ml.

Inoculations. A small amount of inoculum was injected into each of six sites on the underside of a bean primary leaf with a 1-cc plastic disposable syringe with no needle, using one leaf on each of three plants per strain tested. By varying the pressure on the leaf blade between the flat syringe end and a sterile gloved finger, this inoculation method could be carefully controlled to produce uniform water-soaked areas about 4 mm in diameter. A similar method has been recently described (11). Any excess inoculum was carefully blotted from the leaf surface, and the plants were then maintained in a growth chamber at 26 C.

Sampling. Leaf disk samples containing the inoculated area were taken using a 6-mm cork borer on days 0 (immediately after inoculation), 1, 3, and 6. For each of three experiments, three samples were collected per strain each sampling day, one sample chosen randomly from each of the three plants inoculated with each strain. Each sample was placed directly into a sterile 1.5-ml Eppendorf tube containing 50 µl of K buffer.

Tissue maceration. Tissue grinders were prepared from 0.5-ml Eppendorf tubes by removing the caps and top rims with sharp scissors. These were then conveniently sterilized in autoclavable racks designed for P-20 micropipette tips (West Coast Scientific, Inc., Emeryville, CA). By firmly inserting the 6-mm cork borer into

a sterile grinder, the grinder was removed from the rack and inserted into the 1.5-ml Eppendorf sample tube, trapping the leaf disk between the tapered bottoms of the two tubes. This grinding apparatus (Fig. 1) was gently spun between the fingers for 10–20 sec to completely homogenize the leaf disk sample. The used grinder was cleanly released from the cork borer with a poker and discarded directly into an autoclavable waste container.

Bacterial population determinations. The number of colony-forming units per sample was determined using a slight modification of the micro-plating method described by Keen et al (7). After thorough mixing of the sample and rinsing of the tube walls by repeated pipetting, triplicate 5-µl droplets were spotted onto an agar plate. Another 5-µl droplet was then placed into a sterile 0.5-ml Eppendorf tube and diluted 10-fold with 45 µl of K buffer. Triplicate 5-µl droplets of this dilution were spotted as before and the process repeated up to five times as required by strain, with all dilutions for one sample being spotted on a single plate for counting. More recent experiments have used duplicate droplets of each of six dilutions per sample, allowing two samples to be represented on one plate. After 18–36 hr growth at 22 C, plates were observed under a 2× dissecting microscope, and spots containing 15–150 discrete colonies were counted and used to calculate the number of colony-forming units present in the original leaf disk sample.

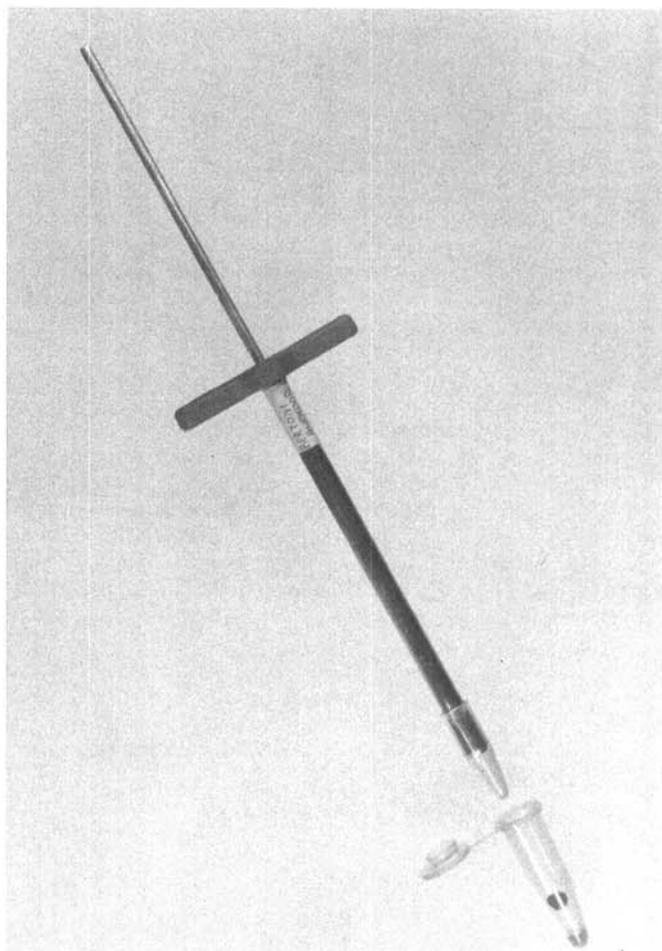


Fig. 1. Tissue grinding apparatus consisting of a 1.5-ml Eppendorf tube containing the leaf disk sample and 50 µl of K buffer (dye added for clarity), a 6-mm cork borer holding a topless 0.5-ml Eppendorf tube, which acts as the grinding head, and an inoculating loop handle inserted through the cork borer as a poker for removal of the used grinder. Best results are obtained when the leaf disk is positioned near the top of the taper of the large tube and the grinder is spun rapidly by twirling the cork borer between the fingers while applying moderate downward pressure.

TABLE 1. *Pseudomonas syringae* pv. *syringae* strains

Strain	Characters ^a	Source or reference
J900	wild type, high virulence	J. Lindemann
PS9020	wild type, moderate virulence; spontaneous sm ^r	(1)
PS9021	PS9020::Tn5, reduced virulence; sm ^r , km ^r	(1)
PS9024	PS9020::Tn5, reduced virulence; sm ^r , km ^r	(1)

^a sm^r/km^r: Streptomycin/kanamycin resistant.

RESULTS

This inoculation technique proved to be a reliable way to introduce a bacterial population of known size into a specific area of leaf tissue with minimal damage to the area inoculated. Because of the well-defined method of inoculum preparation and injection, approximately equivalent numbers of bacteria could be initially introduced into each sample. An experiment was performed to determine the variability in starting populations for a large number of samples, in an attempt to introduce approximately 1,000 cfu (log cfu = 3.0). When 100 sites were inoculated with strain J900 and immediately sampled (Fig. 2), over 50% were found to have log cfu values of 2.9 or 3.0 (approximately 710–1,100 cfu) and 93% had values within 0.3 log (a factor of 2) of the average value of 2.95 (890 cfu). To be certain that all bacteria inoculated remained within the 6-mm sample area, a slightly lesser amount of inoculum was used for the growth experiments, with average starting values for all four strains lying between 2.6 and 2.8, representing 360–700 cfu (Table 2, day 0).

In addition, the grinding apparatus proved to be a clean, efficient way to quickly macerate the leaf disk samples. On each sampling day, the 12 samples could be ground in about 5 min, and on one occasion 100 samples were homogenized in less than 1 hr.

The versatility of this leaf inoculation, sampling, and tissue grinding procedure was demonstrated in a variety of other crop plants by introducing and isolating approximately 10^3 cfu per inoculated site. Over 90% of triplicate samples from tobacco, tomato, potato, lettuce, pea, onion, and radish were found to have log cfu values of 2.8–3.3 (630–1,900 cfu), with the average for all samples being 3.1, or approximately 1,200 cfu (data not shown).

Results of the comparative growth studies are given in Table 2. By day 1 postinoculation, wild-type strains J900 and PS9020 and also mutant PS9024 showed dramatic population increases, whereas mutant PS9021 showed a slight decrease. Strain J900 grew most quickly and to the highest level, consistently reaching greater than 10^7 cfu per sample by day 6. At this point the tissue was totally dry and necrotic, yet very high numbers of viable bacteria could still be isolated. It appeared that 10^8 cfu of these bacteria represented the upper population limit of this size sample.

Strain PS9020, the parental strain from which the two mutant strains were derived through Tn5 mutagenesis, grew less rapidly than J900 and reached a plateau population about two logs lower. This strain also caused a less severe disease reaction than J900 on this bean cultivar, characterized by a less extensive development of necrotic lesions without as much tissue distortion.

No increase in population levels was observed with mutant strain PS9021, which showed a complete lack of ability to induce necrotic symptoms. The only reaction observed was a faint bruising of the inoculated spot, similar to the reaction caused by a control inoculation with K buffer.

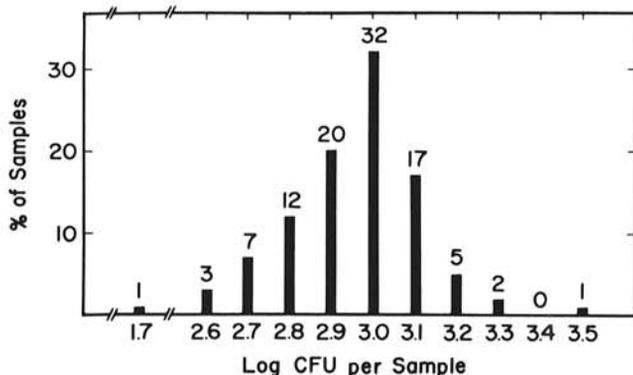


Fig. 2. Variability in initial populations of 100 leaf disks sampled immediately after inoculation with *Pseudomonas syringae* pv. *syringae* strain J900 as described in Materials and Methods.

A similar lack of necrotic symptoms was observed when inoculating with PS9024, even though its initial growth rate was comparable to, or in excess of, that of the wild-type parent PS9020. Although rapidly reaching high population levels by day 3, this strain showed a consistent inability to maintain these levels, as populations by day 6 had always decreased by one or two logs. This was in contrast to PS9020, whose population levels remained stable at day 6.

DISCUSSION

This inoculation and sampling method was found to give results that enabled the reproducible identification of growth patterns in bean leaves for the four strains tested. Triplicate samples on a particular sampling day were usually found to vary from the average by less than 0.5 log cfu, and similar samples in different experiments by less than 1.0 log. These minor differences did not obscure the repeatedly observed growth patterns, which could involve population increases of three to five logs. A twofold error in number of colony-forming units inoculated (0.3 log) would be compensated for by a single bacterial doubling, and a strain that increases three to five logs would have doubled 10–17 times.

This procedure demonstrated that the two wild-type strains and mutant PS9024 could grow rapidly in planta within 24 hr and that mutant PS9021 lacked this growth ability. Currently this technique is being used to study the ability of these bacteria to spread through leaf tissue by sampling tissue surrounding the usual 6-mm sample.

This inoculation, sampling, and tissue grinding procedure was used successfully with bean and other crop leaves to introduce and isolate approximately 10^3 cfu per inoculated site. The necessary materials are inexpensive, easily obtained and sterilized, and disposable. Tubes and grinders are easy to autoclave after use, a consideration that becomes especially important when working with highly virulent organisms or ones that have been genetically engineered. Aside from direct studies of population growth, this technique should prove useful in the study of other molecular and cellular aspects of the host-pathogen interaction including hypersensitivity, plasmid and cosmid stability, genetic complementation, and differential gene expression in planta.

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TABLE 2. Log cfu per sample at various times postinoculation^a

Strain/ Experiment	Day 0	Day 1	Day 3	Day 6
J900/1	2.6	6.5	7.2	7.1
2	2.7	6.5	7.7	7.2
3	3.0	7.1	7.8	7.6
avg	2.8 ± 0.2	6.7 ± 0.4	7.6 ± 0.4	7.3 ± 0.3
PS9020/1	2.6	4.9	4.7	4.9
2	2.8	4.5	6.9	6.9
3	2.8	4.5	5.2	4.9
avg	2.7 ± 0.1	4.6 ± 0.3	5.6 ± 1.3	5.6 ± 1.3
PS9021/1	2.1	1.6	1.4	1.2
2	2.9	1.8	2.6	2.1
3	2.8	2.6	2.5	1.1
avg	2.6 ± 0.5	2.0 ± 0.6	2.2 ± 0.8	1.5 ± 0.6
PS9024/1	2.5	4.2	5.5	4.1
2	3.0	5.5	6.8	5.3
3	2.9	6.0	7.1	4.7
avg	2.8 ± 0.3	5.2 ± 1.0	6.5 ± 1.0	4.7 ± 0.6

^a Values for each experiment are the average of three leaf disk samples per sampling day.

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